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Potassium deficiency affects the carbon-nitrogen balance in cotton leaves

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Abstract: Potassium (K) plays important roles in the metabolism of carbon (C) and nitrogen (N), but studies of K deficiency affecting C-N balance are lacking. This study explored the influence of K deficiency on C-N interaction in cotton leaves by conducting a field experiment with cotton cultivar DP0912 under two K rates (K0: 0 kg K₂O ha⁻¹ and K67: 67 kg K₂O ha⁻¹) and a controlled environment experiment with K-deficient solution (K1: 0 mM K⁺) and K-sufficient solution (K2: 6 mM K⁺). The results showed that leaf K content, leaf number, leaf area, boll number, reproductive dry weight and total dry weight were significant lower under K deficiency (K0 or K1). Lower total chlorophyll content and Chl a/b ratio, and decreased *Pn* along with lower *Gs* and higher *Ci* were measured under K deficiency, suggesting that the decrease in *Pn* was resulted from non-stomatal limitation. Leaf glucose, fructose, sucrose and starch contents were higher under K deficiency, because lower sucrose export was detected in phloem. Although leaf nitrate and ammonium contents significantly decreased, free amino acid content was increased by 40-63% under K deficiency, since lower amino acid export was also measured in phloem. K deficiency also induced lower soluble protein content in leaves. Leaf ATP level was significantly increased under K deficiency, indicating ATP utilization was lower, so that less energy was supplied to C and N metabolism. The ratio of soluble sugar to free amino acid and the C/N ratio markedly increased under K deficiency, and one reason was that the phloem export reduced more prominent for sucrose (54.6-78.0%) than amino acid (36.7-85.4%) under K deficiency. In addition, lower phosphoenolpyruvate carboxylase activity limited malate and citrate biosynthesis under K deficiency, causing a decrease of C flux into the amino acids, which was not beneficial for maintaining C-N balance. Sucrose phosphate synthase and nitrate reductase activities were lower under K deficiency, which would limit sucrose biosynthesis and nitrate assimilation. This was

another factor altering soluble sugar to free amino acid ratio and C/N ratio in the K-deficient leaves.

Keywords: Cotton (*Gossypium hirsutum* L.) leaves; Potassium deficiency; Carbon-nitrogen balance

Abbreviations: PEPCase, phosphoenolpyruvate carboxylase; SPS, sucrose phosphate synthase; NR, nitrate reductase; ATP, adenosine triphosphate; *Pn*, net photosynthetic rate; *Gs*, stomatal conductance; *Ci*, intercellular CO₂ concentration; FW, fresh weight; DW, dry weight; SLW, specific leaf weight

1. Introduction

Potassium (K) is important for ensuring optimal plant growth. Although K is not a constituent of any tissue in plants, it is the most abundant inorganic cation, comprising up to 10% of a plant's dry weight (White and Karley, 2010). K plays important roles in numerous physiological and metabolic processes, like maintenance of transmembrane voltage gradients, cation-anion balance (White and Karley, 2010), osmotic potential and water uptake (Kaiser, 1982), regulating the movement of stomata (Humble and Raschke, 1971) and activation of enzymes (Evans and Sorger, 1966). Investigators also reported that K is needed for CO₂ assimilation (Hu et al., 2015) and nitrogen (N) assimilation (Drosdoff et al., 1947).

Cotton (*Gossypium hirsutum* L.) has a higher demand for K to maintain plant growth and fiber development than other crops with determinate growth habits. Many investigators reported that K deficiency resulted in low seed cotton yield and lint yield (Pettigrew, 1999), due to less boll number (Li et al., 2012), lower boll weight (Gormus, 2002) and lower lint percentage (Pettigrew, 1999). K deficiency negatively affected cotton fiber qualities including fiber length, uniformity ratio, fiber strength, and micronaire (Pettigrew et al., 2005). Some studies also indicated that K deficiency would alter biomass accumulation and partitioning (Makhdom et al., 2007) and morphological indices (Gerardeaux et al., 2009). K deficiency also affected numerous metabolic processes, such as carbon (C) metabolism and N metabolism. Zhao et al. (2001) found that K deficiency could alter the contents of sucrose and starch in leaves, and the percentages of sucrose and starch accounting for total carbohydrates. The activities of Rubisco related to CO₂ assimilation and cy-FBPase involved

in the first step of sucrose synthesis were markedly reduced by K deficiency (Hu et al., 2015). Drosdoff et al. (1947) reported that K^+ was necessary for N metabolism in plants, because NO_3^- was transported together with K^+ in the xylem (Dong et al., 2004). Hu et al. (2016b) also observed that K deficiency reduced NO_3^- allocation to the subtending leaves of cotton. Thus, K deficiency affected the C and N metabolism in plants. However, a comprehensive understanding of the effects of K deficiency on C-N interaction is lacking.

Carbon metabolism and N metabolism are linked because they share organic C and energy supplied by photosynthetic electron transport, CO_2 fixation or respiration (Huppe and Turpin, 1994). As a consequence, there are strong interactions between C assimilation and N assimilation in metabolic processes and energy levels (Fait et al., 2011). Between C assimilation and N assimilation, the oxaloacetate-malate shuttle system serves as a valve regulation the reduction of CO_2 and NO_2^- , and malate content was closely linked to CO_2 assimilation and NO_2^- reduction (Backhausen et al., 1994). Champigny (1995) observed that three enzymes (PEPCase, phosphoenolpyruvate carboxylase; SPS, sucrose phosphate synthase; NR, nitrate reductase) play crucial roles in the C-N interaction. In addition, an interaction between C and N metabolites is observed, because the loading of amino acids depends on sucrose loading and mass flow in the phloem (Wang et al., 2012), and there is a fixed ratio of sucrose to amino acids in the cytosol of phloem (Cakmak et al., 1994). However, reports of the effects of K deficiency on the interaction between sucrose transport and amino acid transport in phloem are lacking.

Therefore, it was hypothesized that K deficiency would influence C/N balance in cotton leaves and change the export ratio of sucrose to amino acid in phloem. The objectives of this study were (1) to explore the effects of K deficiency on C metabolism, N metabolism and C/N balance in cotton leaves in more detail, and (2) to investigate the effects of K deficiency on the export of C and N metabolites in phloem and its relationship with C/N balance in leaves.

2. Materials and methods

2.1. Experiment design

2.1.1. Field study

A field experiment was arranged at the Lon Mann Cotton Research Station in Marianna, AR ($34^{\circ}5'N$, $90^{\circ}5'W$) in the summer season of 2015. The available K content in soil before sowing was 72.7 mg kg^{-1} which was below levels needed for optimal cotton growth (Oosterhuis, 2002). The

seeds were sowed on May 14 in Marianna. The cotton cultivar DP 0912 was selected and a randomized complete block was arranged with four replications. Two K fertilizer levels (K0: 0 kg K₂O ha⁻¹ and K67: 67 kg K₂O ha⁻¹) were applied at the beginning of flowering stage in reference to our former study (Oosterhuis et al., 2014). Each plot size was 4 m × 15 m with 1 m row spacing, and the plant density was 74,000 plants ha⁻¹. Weed and insect control was conducted as needed and furrow irrigation was applied according to the Arkansas irrigation scheduler program, which is based on soil moisture balance and evapotranspiration.

2.1.2. Greenhouse study

A controlled environment (greenhouse) experiment was established at the Altheimer Laboratory, University of Arkansas. The same cultivar was planted on January 20, 2015 in 2-L pots in two same growth chambers (Conviron PGW36, Conviron Inc., Winnipeg, Manitoba, Canada). The growth chambers were set for a 12/12 h photoperiod, a photosynthetic flux density of 800-850 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a relative humidity of 60% and temperatures of 30/25 °C (day/night). Each growth chamber was arranged with 24 pots and each pot just had one plant. One of the growth chambers was regarded as an experiment repeated. The Hoagland's nutrient solution contained 6 mM K⁺, 2 mM NH₄⁺, 4 mM Ca₂⁺, 2 mM Mg₂⁺, 1 mM Fe³⁺, 3.7 μM Mn²⁺, 0.77 μM Zn²⁺, 0.32 μM Cu²⁺, 7.3 μM Cl⁻, 2 mM PO₄³⁻, 2 mM SO₄²⁻, 46 μM H₃BO₃ and 0.12 μM MoO₃, and all pots were watered every two days with one-quarter-strength K nutrient solution (1/4 strength K concentration in above Hoagland's nutrient solution through substituting NH₄NO₃ for KNO₃) and with deionized water alternately until flowering. Two treatments were established at the beginning of flowering stage, containing (1) a treatment without K in the nutrient solution (K1: 0 mM K⁺), and (2) a control with sufficient K supply (K2: 6 mM K⁺). Pots were re-randomized once a week in each chamber from seed germination to the end of the experiment.

2.2. Sampling and processing

At 4 weeks after first flower (90 days after sowing, August 12), the plants in the K0 treatment have showed severe K deficiency symptoms. Four leaves at the fourth main-stem node from the apex of the plant in each plot in the field experiment were used for the measurement of photosynthetic parameters, then were sampled for the measurement of chlorophyll content by removing five discs (0.75 cm² per disc) in 80% acetone extracts (Lichtenthaler, 1987), then the leaves with petiole were transported on ice to the lab for the analyses of leaf K content,

carbohydrates and N compounds. At 6 weeks after first flower (104 days after sowing, August 26), agronomic traits (height, fruiting branch number, leaf number, leaf area, boll number) were measured. Leaf area was recorded by a LI-3100 area meter (LiCor, Lincoln, NE, USA). The plants above ground collected from one-meter row in each plot were divided into stems (and petioles), leaves, and reproductive organs. Dry matter weights of these parts were recorded after drying at 80 °C for 72 h.

In the greenhouse experiment, at 4 weeks after first flower (80 days after sowing, April 10) four leaves at the fourth main-stem node from the apex of the plant were used for the measurement of photosynthetic parameters and chlorophyll content. Eight leaves were sampled for leaf K content, carbohydrates, N compounds and enzymes determinations. Four leaves were used for collecting phloem exudates. Agronomic traits and dry matter weight of plants were also measured with four replications at 6 weeks after first flower (94 days after sowing, April 24).

2.3. Photosynthetic parameters

Before sampling the leaves at the fourth main-stem node from the terminal of the plant, photosynthetic parameters including net photosynthetic rate (P_n), stomatal conductance (G_s) and intercellular CO_2 concentration (C_i) were determined at 9:00-11:00 with a CI-340 hand-held portable photosynthesis system (CID Bio-Science, Inc., Camas, WA, USA) for the field experiment and the greenhouse experiment. Ambient air in the leaf chamber was maintained at 30 °C, relative humidity was 60% and CO_2 concentration of the incoming air was ambient CO_2 concentration.

2.4. Carbohydrates, N compounds, malate, citrate and adenosine triphosphate (ATP) measurements

For field and greenhouse experiments, carbohydrate contents were extracted and assayed according to Loka and Oosterhuis (2016) with slight modification. 40 mg of dried tissue was heated in 1 mL of 80% (v/v) ethanol at 80 °C three times. After combining the three supernatants, 80% ethanol was added into the combined supernatants to a final volume of 3 mL. 30 mg of activated charcoal was added to remove substances that could interfere with the carbohydrate measurements. The supernatant was used for measuring sucrose, glucose and fructose contents after centrifuging at 10,000×g for 15 min. 20 µL of extract was added to a 96-well microtitration plate and the plate was put into a dryer to evaporate the ethanol. 20 µL distilled water was added into each well and the plate was incubated three consecutive times at 30 °C for 15 min with 100 µL glucose assay reagent

[glucose (HK) assay kit; Sigma Chemical Company], at 30 °C for 15 min with 10 µL phosphoglucose isomerase (0.25 EU, Sigma P-9544), and at 30 °C for 60 min with 10 µL invertase (83 EU, Sigma I-4504). After each incubation time, the absorbance was determined at 340 nm by a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). The ethanol-insoluble residue was used for starch extraction. 0.5 mL KOH (1 M) was added into the samples before heating at 100 °C for 1 h. After cooling, β -amylase and amyloglucosidase were added in order to hydrolyze starch. The samples were centrifuged at 10,000×g for 15 min, and the supernatant was collected and diluted by deionized water to 3 mL. Each sample extract (20 µL) was added into a 96-well microtitration plate to determine glucose concentration according to the method described above. The starch concentration was calculated according to Zhao et al. (2008).

For field and greenhouse experiments, nitrate (NO_3^-) was extracted and measured according to Ruiz and Romero (2002). Dried leaves (0.2 g) were extracted with 10 mL Millipore-filtered water. 100 µL extract was taken into a tube and 0.2 mL of 10% (w/v) salicylic acid in sulphuric acid was added. After 20 min, 4.75 mL of 8% NaOH was added into each sample. After cooling to room temperature, the absorbance was measured at 410 nm. Ammonium content was measured as described previously (Lin and Kao, 1996) with slight modification. Leaf samples (0.2 g) were extracted with 3 mL of 0.3 mM sulphuric acid (pH 3.5). Then the samples were centrifuged at 29,000×g for 15 min. Clear supernatant (200 µL) was diluted by 0.3 mM sulphuric acid to 4 mL. For the reaction, 0.5 mL of solution A (5 g phenol and 25 mg nitroprusside were dissolved in 100 mL water) and then 0.5 mL of solution B (2.5 g NaOH were added into 40 mL of 5% sodium hypochlorite and then diluted by distilled water to 100 mL) were added. Incubation was carried out in a water bath at 37 °C for 20 min. The absorbance was determined at A_{625} nm against the control without extract. Ammonium contents were expressed as $\mu\text{mol g}^{-1}$ dry weight (DW). The extraction of free amino acid was the same as the extraction of carbohydrate contents. Free amino acid was measured using the ninhydrin method (Yemm et al., 1955) and was expressed as mg g^{-1} dry weight (DW). Fresh leaf (0.3 g) was used for the extraction of soluble protein according to Hu et al. (2016b). The soluble protein content was determined by Bradford reagent according to a previous study (Bradford, 1976) using bovine serum albumin as a standard.

The malate content of the samples from field and greenhouse experiments was assayed according to Crecelius et al. (2003). 0.2 g fresh leaves were crushed in 1 mL 10% (w/v) perchloric

acid with 20 mg polyclar AT. The homogenate was centrifuged at 4,400×g for 5 min at 4 °C, and then 2% (w/v) perchloric acid was added and the samples were centrifuged again. The two supernatants were pooled and neutralized using 5 M KOH in 1 M ethanolamine. After adding 7% (w/v) polyclar AT and incubation at 4 °C for 30 min, the mixture was centrifuged at 4400×g for 10 min at 4 °C. The malate content was measured by monitoring NAD reduction at 340 nm. The reaction buffer (980 µL) contained 84.5 mM glycylglycine with a pH of 10.0, 0.5 mM NAD, 43.0 mM glutamate, 3.0 units glutamate- oxaloacetate transaminase and 3.0 units malate dehydrogenase. 20 mL extract was added into the buffer to start the reaction.

For field and greenhouse experiments, citrate extraction and assay were according to Moellering and Gruber (1967). 0.5 g fresh leaves were ground in 10 mL of 0.6 M perchloric acid. After centrifugation, the supernatant was neutralized using 2 mL of 2 N KOH and then kept in an ice bath for 15 min. After centrifugation again, the supernatant was used for the determination of citrate content. The reaction solution contained 2.00 mL of 0.1 M triethanolamine (pH 7.6), 0.01 mL of 0.03 M ZnCl₂, 0.06 mL of 0.01M NADH and 0.1 mL extraction. The absorbance was monitored at 366 nm for 5 min.

The ATP content of the samples from greenhouse experiment was determined according to a previous study (Loka and Oosterhuis, 2016). Three leaf discs per leaf were sampled using a cork borer (1 cm in diameter), then three discs were put into a centrifuge tube with 5 mL of 50 mM Tris-HCl solution (pH 7.3). The tubes were heated at 100 °C for 10 min before centrifuging at 21,000×g for 10 min. The supernatant was collected for the quantification of ATP. The firefly luciferin-luciferase assay method (ATP bioluminescent assay kit; Sigma, ST. Louis, MO, USA) was used and luminescence was measured with a 20/20n Luminometer (Turner Biosystems Inc., Sunnyvale, CA, USA). Luminescence of samples compared with that of standards having known ATP concentration, and ATP content was expressed as µg g⁻¹.

2.6. Enzyme extraction and analysis for the greenhouse experiment

Sucrose phosphate synthase (SPS, E.C. 2.4.1.14) was extracted as described previously (Huber and Israel, 1982) with slight modification. The reaction solution (350 µL) containing 50 mM of extraction buffer, 10 mM of MgCl₂, 50 mM of UDP-glucose and 50 mM of fructose-6-P was added into the tubes, then 200 µL of enzyme extract was added. The mixtures were incubated at 30 °C for 30 min. 100 µL of NaOH (2 N) was used to terminate the reaction, and the mixtures were heated at

100 °C for 10 min. After cooling, 3.5 mL of 30% HCl and 1 mL of 0.1% resorcin in 95% ethanol were added into the mixtures before heating at 80 °C for 10 min. After cooling to room temperature, the absorbance values were measured at 480 nm (Hu et al., 2016a).

The determination of nitrate reductase (NR, EC 1.6.6.1) activity was according to the method of Ding et al. (2006). Fresh leaves (0.3 g) were crushed with 4 mL phosphate buffer (0.1 M, pH 7.5), then the homogenate was centrifuged for 20 min at 12,000×g and 4 °C. The resulting supernatant (0.4 mL) was added into the tubes, then 0.4 mL nicotinamide adenine dinucleotide (NADH) and 1.2 mL of 0.1 M KNO₃ were added before incubating at 25 °C for 30 min. The blank solution was added with 0.4 mL of 0.1 M sodium phosphate (pH 7.5) in place of NADH. 1 mL sulphanilamide was used to stop the reaction before adding 1% 2,2 N-1-naphthylethylenediamine dihydrochloride (1 mL). After 15 min, the mixtures were centrifuged at 12,000×g for 10 min. The absorbance of the supernatant was detected at 540 nm, and the enzyme activity was calculated from a standard curve made using nitrite nitrogen.

Phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31) was extracted and measured according to Quay and Champigny (1992). Fresh leaves (0.2 g) were ground with 1 mL buffer containing 50 mM Hepes-KOH (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, 12 mM MgCl₂, 2 mM benzamidine and 2 mM L-amino-n-caproic acid. After mixing, the suspension was immediately stored at -80 °C until PEPCase activity analysis. The reaction buffer (950 µL) contained 50 mM Tris-HCl (pH 7.6), 20 µmol NaHCO₃, 130 nmol NADH, 10 µmol MgCl₂, 5 µmol DTT and 1 unit malate dehydrogenase, and 50 µL enzyme solution was added into the reaction buffer. The reaction was started by adding 3.25 µmol phosphoenolpyruvate at 30 °C. The absorbance was measured at 340 nm.

2.7. Leaf K、C and N contents determination

Leaf K content was measured according to Donohue et al. (1992) using atomic absorption techniques. Leaf C content was determined according to Hafsi et al. (2003) using a wet-combustion method. Leaf N content were measured following H₂SO₄-H₂O₂ digestion method of Kjeldahl (Nelson and Sommers, 1972). The K、C and N contents were assayed by the Soils Testing Laboratory, University of Arkansas, Fayetteville.

2.8. Phloem export of sucrose and free amino acid

Phloem exudates were collected using the EDTA-method according to Wang et al. (2012). The

cut ends of the petioles were immediately immersed into 10 mL of 20 mM EDTA solution with a pH of 6 in the dark for 15 min. In order to avoid contamination with xylem exudates, the 10 mL EDTA solution was discarded. Then the leaves along with petiole were washed and transferred to 10 mL fresh EDTA solution (20 mM). The leaves were placed in the dark in an air-tight chamber in high relative humidity and at ambient temperature throughout the collection period. After 5 h, the exudation solutions were collected for measuring sucrose and amino acid concentrations according to the methods described above (Yemm et al., 1955; Loka and Oosterhuis, 2016).

2.9. Data analysis

For the field experiment, data were analyzed using ANOVA test processed by SPSS statistic package Version 17.0 (Hu et al., 2016a). Differences between mean values were determined by the least significant difference (LSD) test. All analyses of significance were made at the $P < 0.05$ level. All figures were drawn by Origin 8.0.

For the greenhouse experiment, there were no significant differences between the results collected from the two growth chambers, so the results from the growth chambers were pooled. All data were subjected to ANOVA test with SPSS statistic package Version 17.0. Means were separated using LSD test at $P = 0.05$. All figures were drawn by Origin 8.0.

3. Results

3.1. Morphological indices

Height and fruiting branch number were little affected by K rate in the field experiment, but height significantly decreased in the K1 treatment relative to K2 treatment (Table 1). Total leaf number and leaf area were markedly reduced by 42.7% and 53.9% in the K0 treatment relative to K67 treatment in the field experiment, and by 30.9% and 21.9% in the K1 treatment compared with K2 treatment in the greenhouse experiment. Boll number, reproductive organs weight and total dry weight were also significant in the K deficiency (K0 and K1) treatments relative to K application treatments (K67 and K2).

3.2. Leaf K content, chlorophyll content and photosynthetic parameters

There were significant differences in leaf K concentration between treatments (Table 2). The leaf K concentration declined under K deficiency, which was 61.7% lower in the K0 treatment than K67 treatment, and was 67.9% lower in the K1 treatment than K2 treatment. There was a significant ($P < 0.05$) reduction in total chlorophyll content of the K-deficient treatments. K deficiency also

significantly decreased the ratio of Chl *a/b*. *Pn* and *Gs* markedly decreased in the K0 treatment relative to K67 treatment and in the K1 treatment relative to K2 treatment (Table 2). However, *Ci* significantly increased under K deficiency. Specific leaf weight (SLW) was significant higher in the K0 treatment relative to K67 treatment for the field experiment and in the K1 treatment relative to K2 treatment for the greenhouse experiment (Table 2).

3.3. Carbohydrate contents and N compounds contents

K deficiency resulted in significant alterations in carbohydrate contents. Fructose content was unaffected in the field experiment (Fig.1B), but was 173% higher in the K1 treatment than K2 treatment in the greenhouse experiment. The contents of glucose (Fig. 1A), sucrose (Fig. 1C) and starch (Fig. 1D) showed significant increases under K deficiency. For the field experiment, the contents of glucose, sucrose and starch were 10.4, 31.9 and 38.4 mg g⁻¹ in the K0 treatment and 5.2, 16.7 and 23.5 mg g⁻¹ in the K67 treatment, respectively. For the greenhouse experiment, the contents of glucose, sucrose and starch were 10.5, 29.1 and 40.2 mg g⁻¹ in the K1 treatment and 5.0, 14.4 and 23.7 mg g⁻¹ in the K0 treatment, respectively. Leaf nitrate content (Fig. 1E) and leaf ammonium content (Fig. 1F) were significant lower in the K0 treatment relative to K67 treatment and in the K1 treatment compared with K2 treatment. Nevertheless, this trend was not observed in free amino acid content, because free amino acid content significantly increased under K deficiency (Fig. 1G). Similar to leaf nitrate and ammonium contents, soluble protein content was significantly reduced by K deficiency (Fig. 1H), and a decrease of 29 to 36% was observed under K deficiency. K deficiency significantly increased the ratio of soluble sugar to free amino acid by 30-34% (Fig. 2A). C/N ratio was significant higher under K deficiency (Fig. 2B).

3.4. Leaf malate, citrate and ATP contents

Leaf malate content was significantly affected by K deficiency. A decrease of 65% in leaf malate content was observed in the K0 treatment relative to K67 treatment and a decrease of 70% was observed in the K1 treatment relative to K2 treatment (Fig. 3A). K deficiency also resulted in a significant decrease in leaf citrate content (Fig. 3B). In the present study, leaf samples collected from the greenhouse experiment were used for measuring ATP level. Contrary to malate content, leaf ATP level was markedly increased by 40% in the K1 treatment compared with K2 treatment (Fig. 4).

3.5. Enzymes activities

Leaf samples collected from the greenhouse experiment were used for the assays of enzymes. Three enzymes (PEPCase, SPS and NR) were very important in the C and N interaction. The activity of PEPCase decreased by 52% in the K1 treatment compared to K2 treatment (Fig. 5A). An even greater decrease (69 %) was observed in SPS activity in the K1 treatment relative to K2 treatment (Fig. 5B). NR activity was 16.9 mg g⁻¹ FW h⁻¹ in the K1 treatment and was 30.8 mg g⁻¹ FW h⁻¹ in the K2 treatment. (Fig. 5C).

3.6. Export of sucrose and amino acid

The export of sucrose was expected to reduce significantly under K deficiency, and the actual level of sucrose export was significant lower ($P<0.01$) in the K1 treatment than K2 treatment based on a unit leaf or a unit leaf FW (Table 3). On the basis of the ratio of sucrose content in phloem to sucrose content in leaf (phloem:leaf ratio) per unit DW, an even greater decrease (78%) in K1 treatment compared with K2 treatment was observed in sucrose export. Consistent with the trend of sucrose export, free amino acid export in phloem was significantly reduced in the K1 treatment relative to K2 treatment, decreasing by 52%, 40% and 66% based on a unit leaf, per unit leaf fresh weight and phloem:leaf ratio, respectively.

4. Discussion

Most of the K in plants is obtained from soil, and soil K deficiency could negatively affect K accumulation in plants and the distribution of K in vegetative organs (Hu et al., 2016a). In the present study, leaf K content in the K deficiency treatments was reduced to 0.62-0.71%, which was obviously lower than critical leaf K levels ($\geq 0.90\%$) in cotton leaves (Oosterhuis and Bednarz, 1997), indicating that the leaves from the plants without K application were under severe K stress. Zhao et al. (2001) reported that K deficiency would alter chloroplast ultrastructure and affect pigment content in leaves. Total chlorophyll content was significant lower in the K0 and K1 treatments (Table 2), supporting their conclusion. However, the change of the ratio of Chl a/b in Table 2 was inconsistent with their conclusion that K deficiency did not change the ratio of chlorophyll a to chlorophyll b. Fritschi and Ray (2007) reported that the relative proportion of chlorophyll associated with the photosystem (PS) II complex and the PS II core reaction center complex decrease with a reduction in the Chl a/b ratio. Lower Chl a/b ratio in the K0 and K1 treatments indicated that K deficiency influenced the light absorption ability of leaf, and disrupted the photochemical reactions of photosynthesis. Thus, decreased P_n was measured under K

deficiency (Table 2). Bednarz et al. (1998) reported that if the non-stomatal limitations dominated the reduction in P_n , a decrease in G_s and an increase in C_i were expected. In the present study, lower P_n accompanied with lower G_s and higher C_i was observed in the K0 and K1 treatments, leading us to speculate that the decreased P_n under K deficiency was mainly caused by non-stomatal limitation. Lower chlorophyll content and Chl a/b ratio under K deficiency supported this speculation. Basile et al. (2003) and Jin et al. (2011) found similar results in their experiment with almond (*Prunus dulcis*) and hickory (*Carya cathayensis* Sarg.), in which the plants were under severe K stress, biochemical factors became the dominant factor in the decrease of P_n .

Previous studies have reported that K deficiency would inhibit the C assimilation in leaves (Zhao et al., 2001; Hu et al., 2015). In the present study, lower P_n under K deficiency supported their conclusion; However, glucose, fructose, sucrose and starch contents were markedly increased under K deficiency (except fructose in the field experiment, Fig. 1A-D). Huber (1984) speculated that the accumulated soluble sugars in the K-deficient leaves of soybean (*Glycine max* Merr.) might be associated with the restricted export of sucrose from source to sink. In the present study, the results presented in Table 3 showed that sucrose export calculated a unit leaf, per unit FW of leaf or the phloem:leaf ratio, was significantly reduced under K deficiency, supporting Huber's speculation. K^+ gradients serving as a mobile energy source energizes phloem loading (Gajdanowicz et al., 2011) and sugar transporter gene (*OsSUT4*) expression was decreased by K starvation (Jin et al., 2012). Thus, lower sucrose export in phloem might be because K deficiency limited the loading of sucrose and the expression of sucrose transport protein (Cakmak et al., 1994). When massive sucrose could not be transferred in time, it was more likely to convert to other sugars (fructose, glucose and starch) accumulating in leaves (Zhao et al., 2001), resulting in high SLW (Table 2). Previous study found that K application could improve N metabolism, leading to an increased assimilation of N in tea (*Camellia sinensis* L) leaves (Ruan et al., 1998). Hu et al. (2016b) also observed that K application was conducive to the accumulations of N and NO_3^- contents in the leaves. Similar results were measured in the present study. Leaf nitrate content was decreased by 30-34% in the K-deficient leaves (Fig. 1E) and leaf ammonium content also was 30-35% lower under K deficiency (Fig. 1F), which was because the absorption and transport of nitrate and ammonium could be inhibited under K stress (Armengaud et al., 2009; Gajdanowicz et al., 2011). Those changes were not beneficial for the biosynthesis of amino acid, but free amino acid content was 40-63% higher in the K-deficient

leaves (Fig. 1G), perhaps because K deficiency accelerated protein degradation to form amino acid (Hu et al., 2016b). In the present study, amino acid export declined by 36.7% or 41.0% in the K1 treatment compared with K2 treatment based on a unit leaf or per unit leaf FW, and reduced more (85.4%) when evaluated using the phloem:leaf ratio, indicating lower amino acid export in phloem was also one of the reasons for high free amino acid content accumulated in the K-deficient leaves. Moreover, high free amino acid content in the K-deficient leaves might be another reason for the high SLW (Table 2). In accordance with our results, Hsiao et al. (1970) observed a significant increase in free amino acid content in the K-deficient leaves of corn (*Zea mays* L.). On the contrary, leaf soluble protein content was significant lower in the K1 treatment than K2 treatment (Fig. 1H), indicating that amino acid could not be successfully used for synthesizing protein under K deficiency, although amino acid accumulated sharply. Hu et al. (2016b) observed that K deficiency would alter the distribution of nitrogenous compounds between amino acids and proteins, and this change was shown as a higher ratio of free amino acid to protein in the K-deficient leaves (0.71-0.81) relative to K-sufficient leaves (0.31-0.37) in the current study. Similarly, Wahab and Abd-Alla (1995) observed that K deficiency significantly decreased protein content in their experiment with faba bean (*Vicia faba* L.).

Photosynthetic apparatuses including Rubisco, stromal enzymes and thylakoid proteins, are highly dependent on N metabolism in leaves, because photosynthesis requires a large amount of N for the incorporation of CO₂ and water into sugars (Champigny, 1995). The reducing power and energy needed for N assimilation mainly depend on C metabolism (Wang et al., 2015). Thus, C metabolism and N metabolism are closely interwoven and compete for organic precursors and reducing power. Hartt (1970) noticed that ATP synthesis would be restricted by K stress in sugarcane (*Saccharum* spp.) leaves. Conversely, Latzko (1965) observed that the levels of ATP were strongly enhanced in the K-deficient barley (*Hordeum vulgare* L.) roots relative to K-sufficient roots. In this work, leaf ATP level was significant higher in the K1 treatment than K2 treatment (Fig. 4). This was probably because that ATP utilization was inhibited more than ATP synthesis, which would affect the potential energy supply for the metabolism of C and N. Amino acids are the main product of N assimilation which will compete with C assimilation for the C skeletons. The result in Fig. 2A showed that the ratio of soluble sugar to free amino acid was markedly increased under K deficiency, indicating that the increase in soluble sugar content was

more pronounced than free amino acid content in the K-deficient leaves, which led us to speculate that the influences of K deficiency were greater on C metabolism than N metabolism. In support of this speculation, higher C/N ratio was observed in the K-deficient leaves (Fig. 2B). Zhang et al. (2014) noticed that high C/N ratio in cotton leaves was conducive to the growth of reproductive organs. However, higher C/N ratio in the K-deficient leaves did not cause higher weight of reproductive organs in our study (Table 1) because sucrose and free amino acid accumulated in K-deficient leaves could not be transferred to other organs. Consequently, other agronomic characters were also altered, including low leaf number, leaf area, boll number and total dry weight (Table 1). Wang et al. (2012) speculated that phloem loading of amino acid depended greatly on sucrose loading and mass flow in the phloem. Additionally, Cakmak et al. (1994) reported that there was a fixed ratio of sucrose to amino acids in the cytosol of phloem. Our results showed that on the basis of a unit leaf or per unit leaf FW, the rate of sucrose transport was 64-65 times greater than the transport rate of amino acid in the K1 treatment, but it was 85-86 times in the K2 treatment, suggesting that compared with amino acid export, the sucrose export in phloem was reduced more significantly under K deficiency. This result could explain a larger ratio of soluble sugar to free amino acid and larger ratio of C to N occurred in the K-deficient leaves and supported our above speculation that the influences of K deficiency were greater on C metabolism than N metabolism. Three enzymes are very important for the C and N interaction, namely PEPCase, NR and SPS, which play important roles in the anapleurotic CO₂ fixation, N assimilation, and sucrose synthesis, respectively (Champigny, 1995). In C₃-plants, PEPCase plays a crucial role in the organic acid biosynthesis by catalyzing the carboxylation of PEP to produce oxaloacetate when the demand for organic acid in amino acid biosynthesis increases significantly (Champigny and Foyer, 1992). Thus, it is a key enzyme of the anapleurotic pathway. In the present study, PEPCase activity was significant lower in the K1 treatment relative to K2 treatment (Fig. 5A), which would cause a decrease of C flux into the tricarboxylic acid cycle, inhibiting organic acid biosynthesis (Champigny and Foyer, 1992). Lower malate and citrate contents measured in the K1 treatment (Fig. 2C) supported this and lower malate and citrate contents would result in less 2-oxoglutarate participating in the process of amino acid synthesis under K deficiency, limiting the amino acid synthesis to affect the C/N ratio in leaves. Armengaud et al. (2009) also found a decrease in malate content in *Arabidopsis* roots under K stress. In addition, SPS and NR activities were significant

lower in the K1 treatment than K2 treatment, because K deficiency limited the expression of *SPS* gene (Li et al., 2011) and *NRT2* gene (Armengaud et al., 2004). Lower SPS and NR activities would limit sucrose biosynthesis and nitrate assimilation under K deficiency, leading us to conclude that this was another factor altering the ratio of soluble sugar to free amino acid and the C/N ratio under K deficiency. In support of our speculation, lower NR activity together with changed C/N ratio was measured in the K-deficient *Arabidopsis* roots (Armengaud et al., 2009).

5. Conclusion

Decreased *Pn* accompanied with lower *Gs* and higher *Ci* was measured in the K-deficient leaves, suggesting that the decreased *Pn* was mainly caused by non-stomatal limitation, which was supported by lower chlorophyll content and Chl a/b ratio under K deficiency. The contents of glucose, fructose, sucrose and starch were markedly increased under K deficiency (except fructose in the field experiment), because decreased sucrose export rate was measured in the phloem. Leaf nitrate and ammonium contents were reduced, which was not beneficial for the biosynthesis of amino acid, but free amino acid content was higher in the K-deficient leaves, since K deficiency resulted in lower export rate of amino acid in the phloem. K deficiency also limited amino acid to produce protein, so lower soluble protein content was observed under K deficiency. Leaf ATP level was significantly increased under K deficiency, because ATP utilization was restricted for the metabolism of C and N. The ratio of soluble sugar to free amino acid and the ratio of C/N were markedly increased under K deficiency, because the sucrose export in phloem was reduced more than amino acid export under K deficiency. Imbalance in C/N ratio and transport rate under K deficiency decreased leaf number, leaf area, boll number, reproductive dry weight and total plant dry weight. In addition, PEPCase activity was lower under K deficiency, which would limit the C flux into the tricarboxylic acid cycle to inhibit malate and citrate biosynthesis, causing a decrease of C flux into amino acid synthesis under K deficiency. SPS and NR were decreased in the K-deficient leaves, which would limit sucrose biosynthesis and nitrate assimilation, which was another reason for altered soluble sugar to free amino acid ratio and C/N ratio in the K-deficient leaves.

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Figure legends

Fig. 1 Effects of K deficiency on (A) glucose, (B) fructose, (C) sucrose, (D) starch, (E) nitrate, (F) ammonium, (G) free amino acid and (H) protein contents of the leaves. For field experiment or greenhouse experiment, columns followed by different letters are significantly different at $P=0.05$ level. All values are means of four replications \pm standard error.

Fig. 2 Effects of K deficiency on (A) soluble sugar to free amino acid ratio and (B) C/N ratio of the leaves. For field experiment or greenhouse experiment, columns followed by different letters are significantly different at $P=0.05$ level. All values are means of four replications \pm standard error.

Fig. 3 Effects of K deficiency on (A) malate content and (B) citrate content of the leaves. For field experiment or greenhouse experiment, columns followed by different letters are significantly different at $P=0.05$ level. All values are means of four replications \pm standard error.

Fig. 4 Effects of K deficiency on adenosine triphosphate (ATP) level of the leaves from the greenhouse experiment. Columns followed by different letters are significantly different at $P=0.05$ level. All values are means of four replications \pm standard error.

Fig. 5 Effects of K deficiency on (A) phosphoenolpyruvate carboxylase (PEPCase), (B) sucrose phosphate synthase (SPS) and (C) nitrate reductase (NR) activities of the leaves from the greenhouse experiment. Columns followed by different letters are significantly different at $P=0.05$ level. All values are means of four replications \pm standard error.

Table 1 Effects of K deficiency on agronomic characters of the plants for field and greenhouse experiments.

Treatment	Height (cm)	Fruiting branch number (no. plant ⁻¹)	Total leaf number (no. plant ⁻¹)	Leaf area (cm ² plant ⁻¹)	Boll number (no. plant ⁻¹)	Reproductive dry weight (g plant ⁻¹)	Total dry weight (g plant ⁻¹)
Field							
K0	83.7a	12.3a	36.5b	1662.5b	11.5b	30.7b	81.5b
K67	93.7a	13.7a	63.7a	3606.1a	20.3a	76.0a	132.1a
Greenhouse							
K1	122.0b	15.8a	45.6b	4673.3b	7.6b	30.2b	93.6b
K2	132.3a	18.6a	66.0a	5981.6a	14.8a	48.7a	123.7a

For field or greenhouse experiment, values followed by a different letter within the same column are significantly different at $P = 0.05$ probability level. Each value represents the mean of four replications.

Table 2 Effects of K deficiency on leaf K concentration, chlorophyll a+b, Chl a/b, net photosynthetic rate (P_n), stomatal conductance (G_s), intercellular CO₂ concentration (C_i) and specific leaf weight (SLW) of the leaves for field and greenhouse experiments.

Treatment	Leaf K concentration (%)	Chl a+b (mg m ⁻²)	Chl a/b	P_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	G_s (mmol m ⁻² s ⁻¹)	C_i ($\mu\text{mol mol}^{-1}$)	SLW (g m ⁻²)
Field							
K0	0.62b	60.9b	2.04b	3.32b	71.85b	405b	86.7a
K67	1.62a	414.4a	2.94a	15.67a	176.71a	369a	67.0b
Greenhouse							
K1	0.71b	108.1b	2.25b	4.78b	83.25b	399a	64.9a
K2	2.21a	459.3a	2.95a	16.49a	223.12a	338b	53.8b

For field or greenhouse experiment, values followed by a different letter within the same column are significantly different at $P = 0.05$ probability level. Each value represents the mean of four replications.

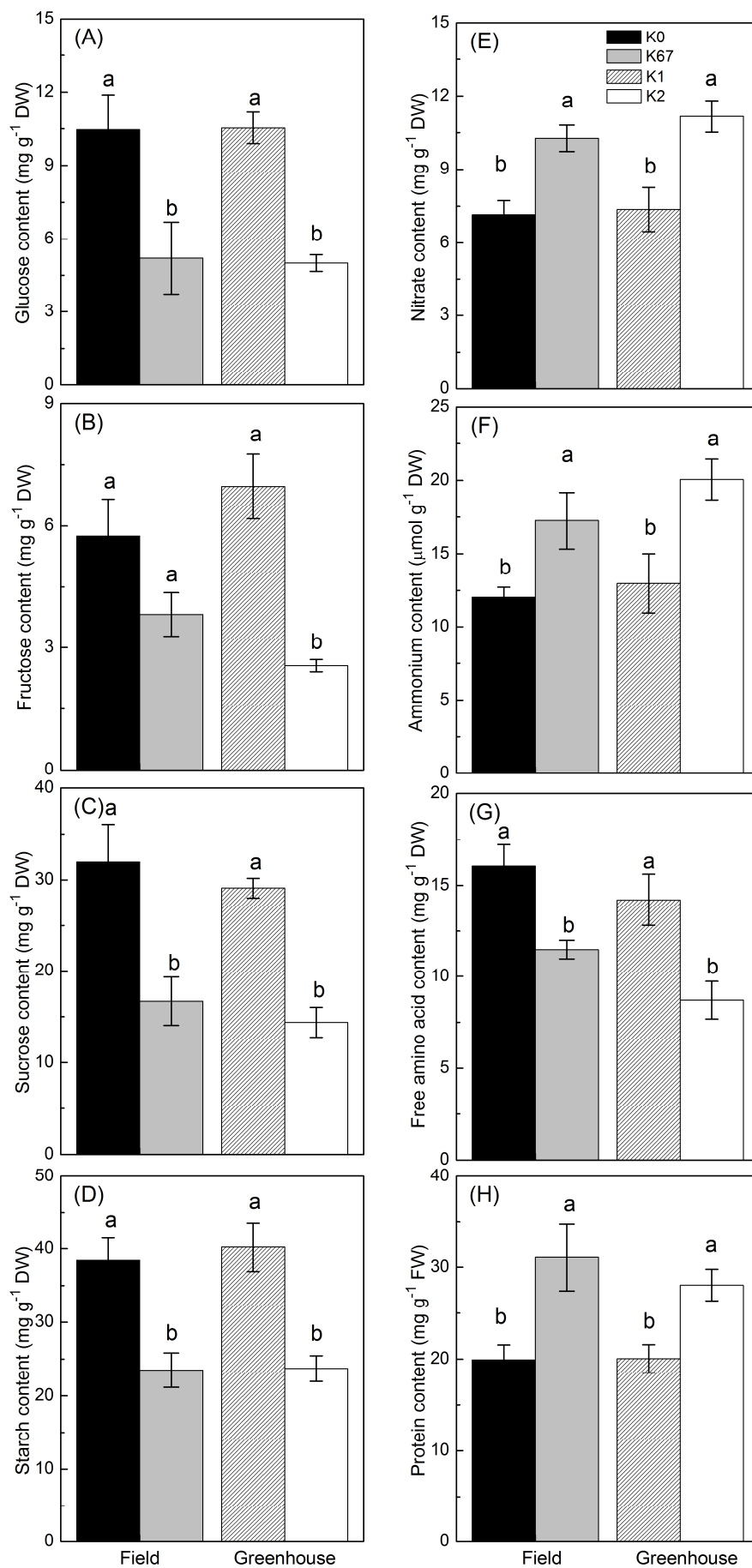
Table 3 Effect of K deficiency on phloem export of sucrose and amino acid for the fourth main-stem leaves from the terminal of the plant for the greenhouse experiment. Phloem exudates were collected from detached leaves using the EDTA-method. All values are means of four replications. ** indicates that the differences between two treatments are significant at $P=0.01$ probability level.

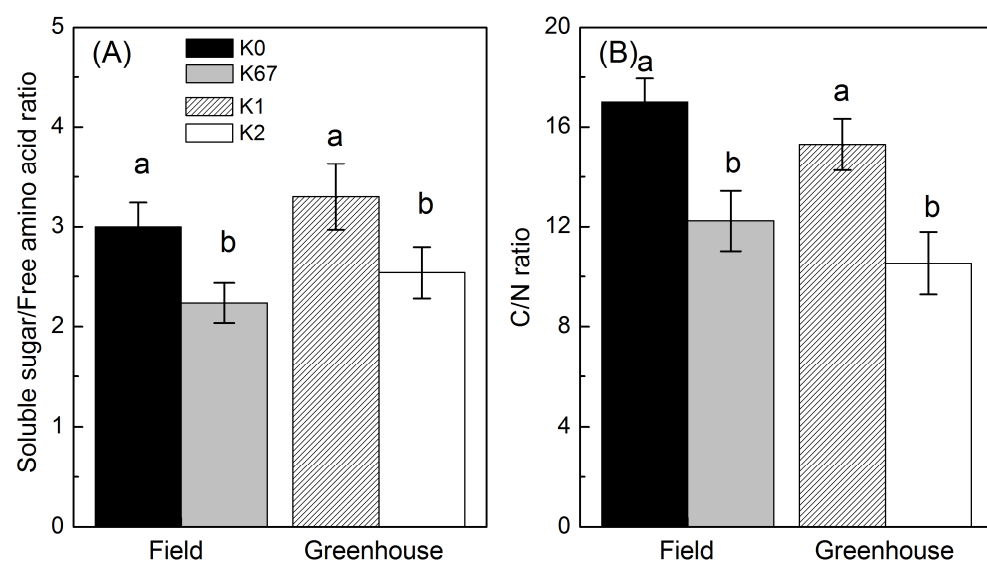
Treatment	Sucrose			Free amino acid		
	Export per leaf ¹ ($\mu\text{g h}^{-1} \text{ leaf}^{-1}$)	Export per unit FW ² ($\mu\text{g g}^{-1} \text{ FW h}^{-1}$)	Phloem/leaf ³ ($\mu\text{g g}^{-1} \text{ FW h}^{-1} / \text{mg g}^{-1} \text{ DW}$)	Export per leaf ($\mu\text{g h}^{-1} \text{ leaf}^{-1}$)	Export per unit FW ($\mu\text{g g}^{-1} \text{ FW h}^{-1}$)	Phloem/leaf ($\mu\text{g g}^{-1} \text{ FW h}^{-1} / \text{mg g}^{-1} \text{ DW}$)
K1	40.6 \pm 3.41	20.2 \pm 2.23	0.695 \pm 0.026	0.621 \pm 0.049	0.311 \pm 0.028	0.022 \pm 0.002
K2	112.3 \pm 2.74	44.5 \pm 5.05	3.163 \pm 0.316	1.303 \pm 0.092	0.521 \pm 0.072	0.064 \pm 0.009
Significance	**	**	**	**	**	**

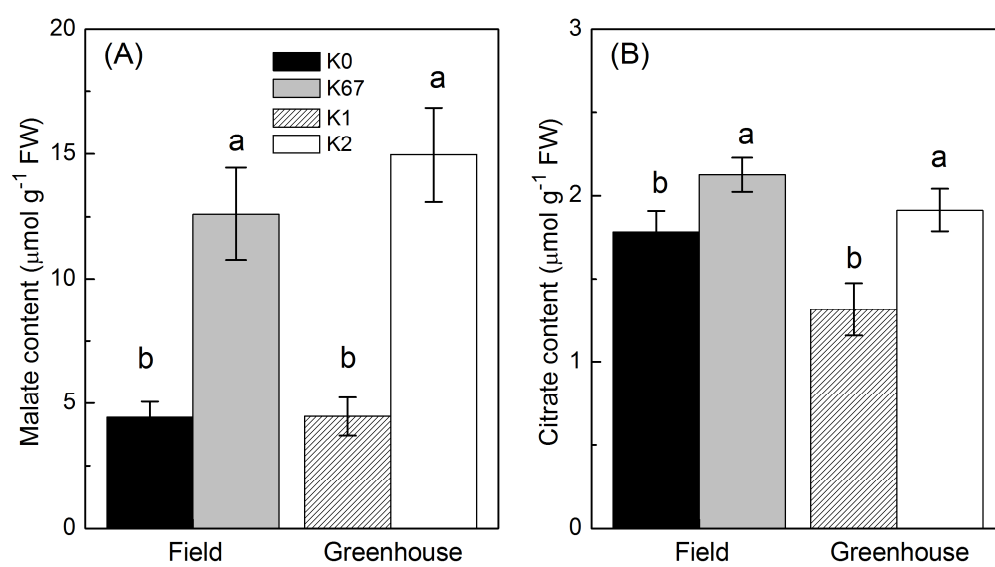
¹ Exported sucrose or free amino acid per hour per leaf.

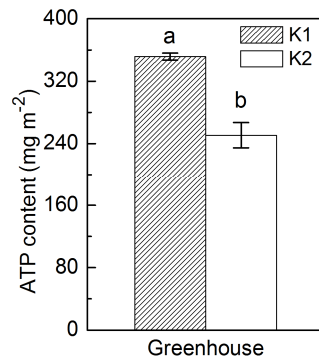
² Exported sucrose or free amino acid per hour per unit leaf fresh weight.

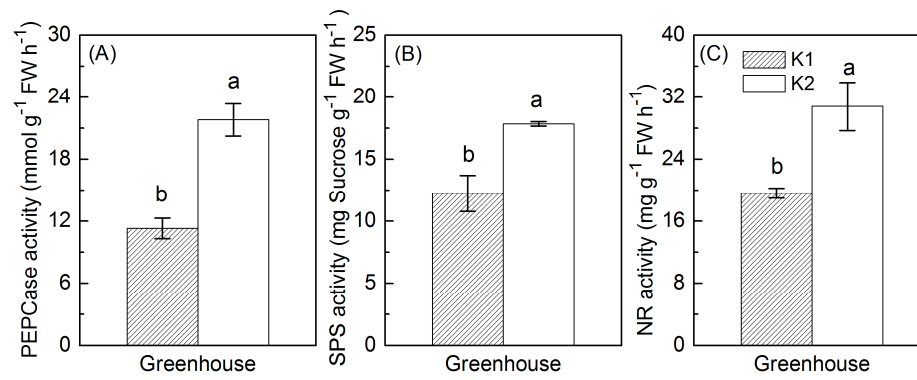
³ Ratio of exported sucrose or amino acid content in phloem exudates to that in leaf per unit leaf dry weight.











Highlights

- (1) K deficiency increased soluble sugar to free amino acid ratio and C/N ratio.
- (2) The sucrose transport in phloem was reduced more than amino acid transport under K deficiency.
- (3) ATP level increased under K deficiency, affecting the energy supply for C and N metabolism.
- (4) Lower PEPCase activity limited malate and citrate biosynthesis under K deficiency, which was not beneficial for maintaining C-N balance.
- (5) SPS and NR decreased under K deficiency, which might be another reason altering C/N ratio.

Contribution

Conceived and designed the experiments: Wei Hu, Derrick M Oosterhuis and Zhiguo Zhou.

Conducted the experiments: Wei Hu and Taylor D Coomer.

Analyzed the data: Wei Hu.

Contributed reagents/materials/analysis tools: Wei Hu, Dimitra A Loka, Derrick M Oosterhuis and Zhiguo Zhou.

Wrote the paper: Wei Hu, Derrick M Oosterhuis and Zhiguo Zhou.